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MICROGLIA – HEALTH PROMOTING PATHWAYS AND THERAPEUTIC TARGETS IN AGEING AND NEUROINFLAMMATION

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To Moa, Edgar, Elis and Emmylou

ABSTRACT

Microglia are the innate immune cells of the CNS with an embryonic origin and self-renew with a slow turnover throughout life. The health-promoting capacities of this cell are being acknowledged through updated and specific tools separating microglia from bone marrow-derived macrophages. Knowing that microglia depends on TGF-β signaling in acquiring a mature homeostatic phenotype, we also found this cytokine to mediate the integration of monocyte-derived cells into an empty myeloid CNS niche. Microglia or the integrated monocyte-derived macrophages, absent in TGF-β signaling, developed a damaging phenotype causing spontaneous de-myelination, clinical motor deficits, and death of experimental mice.

Multiple sclerosis (MS) is a de-myelinating autoimmune CNS disease, commonly with onset in young adults as a relapsing-remitting disease that over time converts to a progressive accumulation of clinical deficits. The etiology of MS is partly inherited but, in many aspects, unknown, although we have successful treatments targeting the adaptive immune system reducing relapses and likely delay progression. However, the progressive MS disease, believed to emanate from the cells residing in the CNS, is very limited in treatment options. Genetic association studies imply that the microglial cell harness a substantial part of the MS-pathogenicity. How this relates to disease phenotypes offering treatment targets is sparsely explored. As the human CNS is rather inaccessible, the use of the rodent animal model experimental autoimmune encephalomyelitis (EAE) has been instrumental in deciphering the MS pathology. In the recovery phase of this disease, the microglial clearance of myelin is of substantial importance. We found this process to depend on a lysosomal degradation process referred to as autophagy- or LC3- associated phagocytosis. During EAE, microglia lacking the autophagy gene *Atg7* accumulated myelin debris and had reduced recirculation of scavenger receptors, causing a secondary impairment in tissue myelin-clearance. These cells also acquired an altered transcriptome associated with inflammatory microglia/macrophage phenotypes found in, e.g., MS, neurodegenerative disease, and stroke, while the genes of the homeostatic signature were downregulated. Autophagy is known to alter with age, and we targeted this by increasing autophagy-associated phagocytosis in aged microglia by treatment with the sugar molecule trehalose, which ameliorated EAE. Of note, trehalose metabolism and some autophagy-associated phagocytosis pathway components are associated with MS through risk allele analysis.

Microglial proliferation and survival rely on CSF-1 (M-CSF) or IL-34 mediated activation of the CSF-1 receptor (CSF1R). While microglial CSF-1 expression is elevated during inflammation, CSF1R associate with the homeostatic microglia. In the healthy CNS, neurons are the main source of IL-34, but a specific role of this cytokine in neuroinflammation remains to be evaluated. The ageing CNS is challenging for microglia in terms of adaptations to aid in health-promoting capacities in functions that, together with CSF1R signaling, engage canonical-autophagy. This degradation pathway controls the quality and quantity of, e.g., inflammatory mediators, receptors, and organelles. By deleting the key canonical-autophagy gene *Ulk1*, we found an age-associated subpopulation of microglia with highly activated ERK1/2 upon CSF1R engagement to be diminished, a loss not compensated by other microglia or myeloid cells. The loss of this population in aged mice caused neural and glial cell death and high mortality in EAE. In autophagy-competent aged mice, we could expand this CNS protective population specifically by IL-34 treatment and thereby ameliorate disease.

In this thesis, I present TGF-β as an essential factor in establishing a homeostatic CNS myeloid cell and a demand in aged microglia for canonical-autophagy to maintain a neuroprotective phenotype. The myelin processing by microglia through autophagy-associated phagocytosis is dissected in detail, and we can show how a decline in this pathway can be restored in aged microglia.

LIST OF SCIENTIFIC PAPERS

I. **Berglund R**, Guerreiro-Cacais AO, Adzemovic MZ, Zeitelhofer M, Lund H, Ewing E, Ruhrmann S, Nutma E, Parsa R, Thessen-Hedreul M, Amor S, Harris RA, Olsson T, Jagodic M.
Microglial autophagy-associated phagocytosis is essential for recovery from neuroinflammation. **Science Immunology**, 5., 52 (2020)

II. **Berglund R,** Guerreiro-Cacais AO, Piket E, Jagodic M, Olsson T. *The ageing CNS is protected from neuroinflammation by an autophagy dependent microglia population promoted by IL-34.* **Manuscript**

III. Lund H, Pieber M*, Parsa R*, Grommisch D, Ewing E, Kular L, Han J, Zhu K, Nijssen J, Hedlund E, Needhamsen M, Ruhrmann S, Guerreiro-Cacais AO, **Berglund R**, Forteza MJ, Ketelhuth DFJ, Butowsky O, Jagodic M, Zhang XM*, Harris RA*.
Fatal de-myelinating disease is induced by monocyte derived macrophages in the absence of TGF-β signaling. **Nature Immunology**. 19(5), 1-7 (2018).
*Equal contribution

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

I. Hochmeister S, Aeinehband S, Dorris C, **Berglund R**, Haindl MT, Velikic V, Gustafsson SA, Olsson T, Piehl F, Jagodic M, Zeitelhofer M, Adzemovic MZ.
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II. Castelo-Branco G, Stridh P, Guerreiro-Cacais AO, Adzemovic MZ, Falcão AM, Marta M, **Berglund R**, Gillett A, Hamza KH, Lassmann H, Hermanson O, Jagodic M.
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CONTENTS

ABBREVIATIONS

ALS	Amyotrophic lateral sclerosis
AMPK	5' adenosine monophosphate-activated protein kinase
ATG	Autophagy related gene/protein
BAM	Border-associated macrophages
BBB	Blood-Brain-Barrier
BMDM	Bone-marrow derived myeloid cell/macrophage
CD	Cluster of differentiation
CLEC	C-type lectin
CNS	Central nervous system
CSF	Colony stimulating factor
CX3CR1	Chemokine(C-X-C motif) 3 receptor 1
DAM	Disease-associated microglia
DCs	Dendritic cells
EAE	Experimental autoimmune encephalomyelitis
ERK	Extracellular signal-regulated kinases
IL	Interleukin
LC3	Microtubule-associated proteins 1A/1B light chain 3
MOG	Myelin Oligodendrocyte Glycoprotein
MS	Multiple sclerosis
MSR1	Macrophage Scavenger Receptor 1
mTOR	mechanistic target of rapamycin
OPC	Oligodendrocyte progenitor cell
PLP	Proteolipid protein
PPMS	Primary progressive MS
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RRMS	Relapsing-remitting MS
SLE	Systemic lupus erythematosus
SPMS	Secondary progressive MS
TAM	Tamoxifen
TGF-β	Transforming growth factor beta
T_H	T-helper cell
TNF	Tumor necrosis factor
TREM2	Triggering receptor expressed on myeloid cells 2
ULK1	Unc-51 like autophagy activating kinas

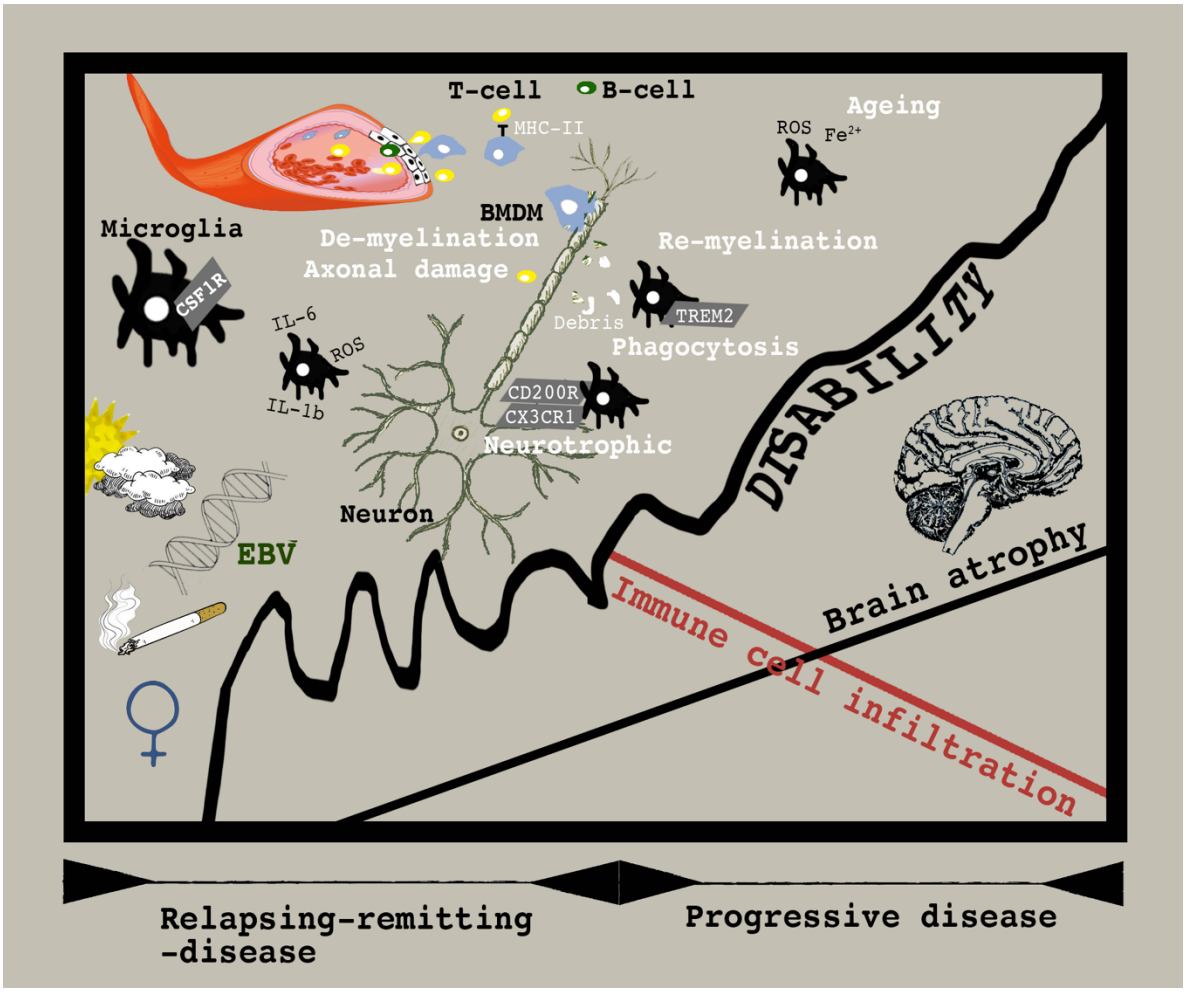
1. INTRODUCTION

1.1 MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a leading cause of neurological disability of young adults, with close to three million cases worldwide and a high and increasing prevalence in northern Europe and the US of about 1 per 750 citizens [1,2]. In MS, an aberrant immune activation towards myelin and neuronal epitopes cause de-myelination and axonal damage leading to neurological deficits determined by the localization of the inflammatory lesion[3]. The variety of signs and symptoms include motor deficits, sensory losses, and cognitive impairment. They commonly appear in the age 20-40 as a Relapsing-Remitting MS (RRMS) in which a recovery phase follows clinical manifestations. In many patients, the recovery declines with age, and the symptoms get chronic as the de-myelinated lesions expand and neuronal/axonal damage accumulate, resulting in Secondary Progressive MS (SPMS). In a small proportion of patients, the recovery is absent already from the onset in a Primary Progressive MS (PPMS)[3]. Charcot first described MS in 1868 as a pathological entity. This was followed by a century of observations before the understanding and treatments of the disease advanced by genetic association studies, animal models, and the development of immune-modulating drugs. The more than 200 genes affecting MS susceptibility are predominantly associated with immunity, and many MS genes are highly expressed in the CNS resident microglial cell with a phenotype shared to a high degree between MS and other neurodegenerative pathologies[4]. Although the overlap of susceptibility risk genes to primary neurodegenerative diseases is small, the MS pathology includes neurodegeneration associated with oxidative stress, mitochondrial dysfunction, and elevated ion-channel activity[5]. Similar to primary neurodegenerative diseases where e.g., α -synuclein and amyloid-beta is fundamental in pathology, the protein Bassoon is recently shown to aggregate in neurons in MS, a pathology found treatable in experimental models[6]. Further, several neurotrophic pathways in myeloid-neuronal interactions, including IGF-1-and CD200R signaling, are highlighted pathways in analysis of MS risk genes[7]. In addition to the inherited genetic risk alleles, female gender, smoking, Vitamin D deficiency, obesity, and Epstein-Barr virus infection are known factors increasing MS incidence, while ageing is the strongest risk factor for disease-progression[8].

The inflammatory MS-relapses' initiating events are likely varied, but the bouts are largely dependent on T-and B-cells specific for CNS-antigens[3,9]. These cells evade tolerance and act synergistically in secondary lymphoid tissues to shape pathogenic T_H1 and T_H17 cells with CNS-migratory potential[9,10]. A break-down of the blood-brain-barrier integrity is an early event in MS relapses and a prerequisite for infiltrating pathogenic peripheral immune cells, including inflammatory monocytes and adaptive immune cells. This is proposed to be an effect derived from CNS resident cells producing e.g., IL-1β and IL-6 and direct leucocyte mediated injury[3]. Today, the inflammatory MS bouts are successfully treated by lymphocyte depletion through monoclonal anti-CD20 or anti-CD52 or by obstructed migration to the CNS by VLA-4 or Sphingosine-1 receptor internalization[3]. These treatments reduce the annual relapse-rate from approximately 0.60 to 0.15[11,12]. However, the MS pathology involves other immune and glial cells and the MS lesions are, besides from CD8+ T-cells, dominated by macrophages of microglial or

monocyte origin[13]. The vast inflammation in MS is mirrored by elevated levels of pro-inflammatory cytokines, e.g., IFN-γ, IL-1β, GM-CSF, IL-17, IL-23, TNF, and CXCL-13[14,15]. Abrogating treatments or gene ablation in animal models targeting each of these cytokines ameliorate disease, but single targeting has not shown to be sufficient to treat human MS[16-27]. In the progressive stages of MS, the BBB is usually more intact, and infiltration of peripheral immune cells causing relapses is rare, while other pathogenic mechanisms such as dysregulated mitochondria and iron-metabolism appear as stronger factors[3,28]. Treatments targeting infiltrating lymphocytes have a limited effect at this disease stage, but CD20+ B-cells, plasma cells, and CD8+ T-cells remain in the CNS and reside in meninges, causing subpial cortical inflammation in interplay with parenchymal glial cells[29-31]. MS-lesions are characterized by the activation state of macrophages and degree of de-myelination, which is prominent in both acute relapse and progressive disease[32]. Innate immunity and target tissue interactions in general and specifically microglial phenotypes are highlighted in analyses of MS risk genes and progressive disease pathology[7,33]. The microglial cell acts as a glial cell with neurotrophic support and as an immune cell with phenotypes associated with the initial events of MS lesions as well as progressive MS[7,13]. This thesis aims to deconvolute and target pathological mechanisms derived from myeloid and microglial dysfunctions. Targeting these cells can potentially abate acute inflammation, but the urgent hopes lie in stimulating recovery, re-myelination, and limit axonal damage in progressive disease[7,13,34,35].



Multiple sclerosis

1.2 RODENT MODELS OF MULTIPLE SCLEROSIS

Since its development by Rivers et al. in the 1930s, the experimental autoimmune encephalomyelitis (EAE) models of MS have been widely used and central in understanding autoimmune de-myelinating inflammation[36,37]. Most of the T-cell-driven pathology and pathognomonic de-myelinating processes with associated axonal damage found in human MS have been mimicked by studies in these models[36,38]. The rodent EAE model is induced either by passive immunization in cell-transfer settings or/and by active immunization with myelin antigens or emulsified spinal cord. The myelin antigens used are complete proteins or peptides with known T- or B-cell immunoreactivity, e.g., MOG. The animal species, strains, and antigens offer a wide variety of clinical courses and reproduce specific aspects of MS pathology. In passive EAE, induction by transfer of CD4+ T-cells is most established, while CD8+ cells are abundant in lesions of both MS and some EAE models and shown to either ameliorate or aggravate disease[39-42]. B-cells cannot transmit passive EAE, but depletion in the C57B/6 mouse MOG 1-125aa model ameliorate disease. The effect on MOG 35-55 peptide induced disease in the same strain is rather aggravating EAE, illustrating that variations in the model can reflect different aspects of human MS[43,44]. Independently of the chosen model, the quantified clinical symptoms are usually an ascending paralysis. Besides the triggered adaptive immune response, the innate immune cells, including microglia, DCs, and monocyte-derived cells, are all fundamental in the EAE pathology, and as the focus of this thesis reviewed in depth below[33].

MS-like disease can also be induced using Theiler or murine corona viruses[38,45]. To specifically study de- and re-myelination, the Cuprizone and the lysolecithin models are widely used where chemical damage of myelin is the initial event[46]. In contrast to EAE, these models are not commonly used for the quantification of clinical motor deficits. In the studies conducted within this thesis, we employ MOG-induced EAE in C57B/6 mice.

1.3 MICROGLIAL ORIGIN AND KINETICS

The microglial cell lineage constitutes about 10% of the CNS cells and is defined by localization, function, appearance, and recently by its yolk-sac derived ectodermal origin and transcriptional profile[34,47,48]. Microglia are commonly considered self-renewing with an annual turnover in the human CNS of about 28% with less than 1% being positive for mitotic labeling in mice and human[47-52]. The establishment and maintenance of the microglia population are dependent on the transcription factors IRF8 and PU.1 and the activity of CSF1R[53,54]. Accordingly, inactivating mutations of this receptor entail a loss of the microglial population in both humans and animal models[49,54,55]. The CSF1R binds two known ligands; CSF-1, expressed primarily by immune cells including microglia, and IL-34, expressed by neurons and glial cells with ~ 300 times higher concentrations compared to CSF-1 during homeostatic CNS conditions[56-58]. IL-34 has two other known receptors expressed at low levels by microglia; Syndecan-1 (CD138) and PTP-ζ (PTPRZ1). The latter suggested to be associated with a neurotrophic phenotype, but neither have a known impact on microglial survival nor proliferation[59-62]. Experimental deletion of IL-34 or CSF-1 genes reduces the population in a region-dependent manner while deleting both genes decreases microglial counts similar to the low density found in mice carrying CSF1R null mutations[49,63-65]. The downstream effects of the CSF1R engagement are complex with the

regulatory activity of several pathways, including NF-κB, ERK, and autophagy steering Akt and AMPK[66,67].

Moreover, to maintain the population, microglia are shown to undergo local stochastic expansion from differentiated cells both in health and disease, and the support for a microglial "stem-cell", distant CNS-migration, or contribution to the population from bone-marrow-derived cells have little support[68-70].

The local renewal of microglia and shaping signals from surrounding tissue gives a cue for spatially defined microglia. IL-34 is associated with the forebrain, hippocampal, and cortical microglia populations, while CSF-1 is necessary for the neurotrophic microglia population in the cerebellum and brainstem[63,64,71]. Microglia density is higher in the IL-34 dependent regions, while microglia in CSF-1 dependent regions are more phagocytic[72,73]. Further, neutralizing anti-CSF-1 antibodies selectively reduces the microglia population in white matter CNS, while anti-IL-34 act stronger on the gray matter population[56]. These regional and possibly functionally altered microglia populations derived from IL-34 or CSF-1 is possibly attributed to variations in CSF1R affinity and binding kinetics[63,74,75]. IL-34 and CSF-1 treatments both ameliorate EAE in a similar fashion, although a moderate but evident cytokine-specific microglial polarizing potential is reported[57,58,75,76].

Repopulating microglia post depletion restore the majority of the expression profile while bone marrow-derived monocytes populating the microglial niche adopt a "semi-microglia" transcriptome supporting a tissue-determined homing cue for a microglial phenotype in addition to the impact from origin[77-80].

1.4 MODELS OF MICROGLIAL DEPLETION

Attempts to deplete microglia and macrophages have a long history for both scientific and therapeutic purposes. In addition to the developmental microglial loss seen in CSF1R/CSF-1/IL-34 deficient mice, microglia can also be successfully depleted using CSF1R/CSF-1/IL-34 inhibitors, clodronate liposomes, or Diphtheria toxin/-receptor or Herpes simplex receptor transgenic approaches[81]. If the depleting condition is released, e.g., withdrawn CSF1R inhibition or loss of induced Diphtheria toxin expression, the microglial myeloid CNS niece is rapidly reconstituted[78,80,82]. This restored population's origin is a mixture of infiltrating bone marrow-derived myeloid cells and expansion of the microglia that evaded depletion[78,80]. Another strategy described but rather unexplored is a transfer of cytotoxic CD8+ T-cells reactive to the GFP expressed in CX3CR1-GFP mice, causing an almost complete microglial depletion[83].

Up to date, there is no post-developmental microglia-specific depletion since the strategies presented above target bone marrow-derived macrophages and are further complicated by a "cytokine storm" and infiltration of peripheral immune cells following depletion[84,85]. The clinical outcome upon microglial depletion in neuroinflammatory and neurodegenerative disease models is mixed which likely reflects the unsolved methodological obstacles[76,86-92].

1.5 MICROGLIAL PHENOTYPES

1.5.1 Homeostatic microglia and tissue interactions

In healthy conditions, the homeostatic microglia act as a glial cell with trophic neuronal support interacting with astrocytes and cells of the oligodendrocyte lineage, and in the clearance of tissue debris important in keeping inflammatory processes in check[93]. The homeostatic microglia are characterized in mice by expression of, e.g., *P2ry12, Cx3cr1, Sall1,* and *Tmem119*[72,94]. The human microglia is more diverse, but the homeostatic population defined by the signature expression of *P2RY12*, *TMEM119*, and *CX3CR1* is found both by single-cell RNA sequencing and mass cytometry[72,95,96]. In addition to the homeostatic core, microglia are further transcriptionally determined by the localization and tissue conditions such as age[72,95-97].

1.5.2 TGF-β

The cytokine TGF-β1 (Hereafter referred to as TGF-β) and its signaling through the TGFBR (composed of the TGFBR1 and 2 subunits) is essential in acquiring a microglial phenotype and the homeostatic transcriptome[98,99]. The TGF-β derived signature is among CNS myeloid cells specific for microglia, and congenital ablation of *Tgf-β* entails a loss of this CNS parenchymal myeloid population[100]. TGF-β loss does not impair cell survival in mature microglia but alters the activation to a pro-inflammatory phenotype[98,99]. In neuroinflammation, TGF-β acts in dampening inflammation and promoting re-myelination by stimulating myelin phagocytosis in EAE[98,101-103]. Further, TGF-β is shown to stimulate myelin phagocytosis by human macrophages in vitro[102]. TGFBR2 deletion causes, besides the diminished homeostatic microglial phenotype, an activating phosphorylation of transcription factor TAK1 regulating microglial derived inflammation[99,104].

Thus, it is evident how TGF-β has a considerable health-promoting potential to be further explored in experimental and human diseases.

1.5.3 Microglial phenotypes in CNS pathology

Upon challenges such as neuroinflammation and neurodegeneration, the genes defining the homeostatic microglia are downregulated, and an activated phenotype collectively referred to as Disease-associated microglia (DAM) is acquired[4]. These phenotypes are characterized by a changed transcriptome association with various CNS disorders and models, including MS, ALS, and other neurodegenerative conditions[4,72,94,105]. There is no unified subset of "DAM core-genes", but in many models, these transcriptomes are dependent on TREM2 signaling[4,72,94,105].

Studies of EAE and experimental neurodegeneration define DAM populations enriched for, e.g., *Ly86*, *Clec7A*, *Apoe*, *Ccl-2,* and *Lyz2* accompanied by reduced expression of homeostatic genes such as *P2ry12* and *Tmem119*[94,105,106]. In MS, several DAM populations are identified, including a phagocytic population with an enriched expression of lysosomal component *Lamp1* and suggested to be key in re-myelination[72,107]. Microglia found in human active MS lesions are commonly positive for macrophage markers such as p22phox, CD68, CD86, MHC class II molecules, and intracellular myelin, while anti-inflammatory markers such as CD206 and CD163 are prominent on microglia associated to inactive lesions[13,103 108]. These markers are lost on Tmem119+ microglia from the chronic

lesion associated with progressive MS [13,108,109]. Recent findings show active de-myelinated lesions microglia to stain for MERTK, TIM-3, and LAMP1, with regulatory and functional implications in phagocytosis[108]. The DAM phenotypes associated with diseases are composed of many microglial transcriptional cues for functions that remain to be explored.

1.5.4 Phagocytosis and re-myelination in MS and EAE

Microglia are CNS resident macrophages, and phagocytosis is a key feature of both DAM and homeostatic phenotypes. Phagocytosis is the endocytic process where extracellular content is engulfed by receptor-mediated budding of the plasma membrane and degraded after fusion with a lysosome. The term macrophage describes an innate immune cell's phenotype specialized in phagocytosis but does not state their ontogeny. In MS and EAE, microglia are the paramount phagocyte of apoptotic cells and myelin debris generated by inflammation, while the damaging de-myelination is executed to a larger extent by monocyte-derived macrophages (also referred to as BMDM)[101,110-115]. Clinical deficits in MS and EAE correlate to de-myelination of the axons, and re-myelination both protect the axons from further damage and restore the signal transduction, thus recovering function[116,117]. The re-myelination sheets are traceable as they are shorter and thinner, but the dynamics of the opposing de- and re-myelination are challenging to study in human disease[116,117].

In the de-myelinating cuprizone and corona-virus MS-models and in EAE, re-myelination depends on the microglial clearance of tissue myelin debris, allowing differentiation of Oligodendrocyte-progenitor-cells (OPCs) into myelinating Oligodendrocytes (OLs)[87,113,118,119].

1.5.5 The receptors regulating and executing microglial phagocytosis

Most significant in dictating the phagocytic microglial phenotype in models of MS, Alzheimer´s, and Parkinson´s is the TREM2 receptor[94,105,111,120-122]. In EAE, gene ablation or antibody-mediated blockade of TREM2 aggravates EAE while stimulation with an agonistic antibody increases myelin clearance and hence, OPC differentiation and re-myelination[111,123-125]. In humans, mutations in the TREM2 gene cause microglial dysfunction and the neurodegenerative Nasu-Hakola disease[126,127]. TREM2 recognizes phosphatidylserines. These lipids are found abundantly in myelin, and apoptotic cell membranes, and binding not only increases uptake but also stimulates the degradation of phagocytosed content[126,128-131]. Degradation of debris and apoptotic cells is further shown to be regulated by the TAM receptors - *MerTK* enriched in the homeostatic TGF-β dependent microglia population, and *Axl* enriched in DAM[72,94,105,132,133]. The uptake of myelin is executed by scavenger receptors MSR1 (SR-A), CR3, CD36, and possibly by TREM2 directly[128,131,134,135].

The myelin phagocytosis is not only an executive function of the health-promoting microglia. It is also evident how accomplished ingestion and myelin degradation further induce a beneficial microglial phenotype in MS and EAE. This phenotype is defined by transcriptome analysis and immunohistochemistry defined qualities, including secretion of TGF-β and IGF-1, a mediator central in OPC differentiation to myelinating OLs[101,136,137].

1.5.6 APOE and CLEC7A

Microglia exposed to apoptotic cells upregulates *Apoe* and *Clec7a* (Dectin-1) genes in transitioning from a homeostatic to a phagocytic DAM phenotype[94]. APOE serves as a transporter of lipids and as a ligand for intracellular receptors such as the LXR[131]. This intracellular receptor and transcription factor mediate lipid efflux and initiate lipid degradation through increased expression of *Apoe* in a positive feed-back loop[131,138,139]. The *APOE* gene contains the strongest risk allele for Alzheimer´s disease and is, through binding TREM2, essential for DAM differentiation[94,140]. The risk-variant *APOEe4* associates with dysregulated phagocytosis, exemplifying an errant DAM phenotype driving pathogenesis[140-142]. Carrying the *APOE4* risk allele does not associate with MS susceptibility but is suggested to accelerate disease progression[143-145].

As part of a microglial phenotype enriched for genes promoting phagocytosis and phagosome degradation, *Clec7a* associates with microglia important in mouse and human development[146,147]. This microglial population is absent in the adult CNS at homeostatic conditions but shows similarities to the TREM2-dependent DAM microglia[94,105,146,147]. The CLEC7A is commonly known to bind fungal cell wall compounds but can bind apoptotic cells during inflammatory conditions[148]. Ablation of the *Clec7a* gene is suggested to aggravate EAE, and activation of CLEC7A by the β-glucan Zymosan promotes axonal regeneration through an ERK1/2 pathway[149-151].

1.5.7 Microglia in neurogenesis and microglial neurotrophic features

Microglial phagocytosis is not only a fundamental health-promoting process in the CNS during inflammation. From embryogenesis, during development, and in the adult CNS, these cells phagocytose apoptotic neural lineage cells and synapses in a process supportive of neurogenesis, myelination, and synaptic plasticity[152-155]. This process relies on the microglia population sustained by CSF1R activity and expression of the fractalkine receptor (CX3CR1), key in microglial motility and spatial orientation mediated by neuronal CX3CL1[154,156]. The synaptic pruning in neuronal plasticity is shown dependent on activation through CR3 and TREM2[157,158]. On the negative side, in the aged CNS, excessive synaptic clearance by complement receptor-activated microglia associate with cognitive decline[159,160]. Neuronal-microglial interaction through CD200-CD200R stabilize microglia and possibly act trophic on neurons[161]. Dysregulation in this and the CX3CL1-CX3CR1 communication is found in MS lesions, Alzheimer´s disease and in the ageing brain[161,162]. Disruption of the CX3CR1 signaling also attenuates experimental neuroinflammation, although this effect is not defined as specifically microglia derived[163,164].

Pathway analysis of the risk-genes associated with MS highlights the IGF-1, CNTF, BDNF, EGF, IL-10, and NGF neurotrophic pathways, all mediators secreted by microglial cells during inflammation[7]. Of certain interest is *Igf1* found enriched in DAM, especially in the phagocytic microglia population associated with EAE recovery, the anti-inflammatory cytokine IL-10 secreted upon phagocytosis, and BDNF with a known age-associated decline[101,105,165-167]. Microglia is in addition to this neurotrophic support shown to recruit neuronal progenitors in the adult CNS in response to tissue damage[168].

1.5.8 Damaging and pathogenic microglial processes

Microglia exhibits damaging potential through cytokine and ROS/RNS secretion during inflammation or neurodegeneration, and microglial depletion can ameliorate pathology[90,91]. During neuroinflammation, ROS is generated in myeloid cells by myeloperoxidases, NOX-complexes, and mitochondria[169,170]. Errant ROS production is an early and pathogenic event in autoimmune neuroinflammation and is counteracted by redox mechanisms, e.g., NRF2 mediated expression of antioxidants[13,170-172]. Moreover, catalase treatment reduces ROS and stabilizing the mitochondria, protects the axons, and ameliorates EAE[173-176]. Extracellular ROS is damaging myelin and axons and acts on the BBB to increase permeability[177,178]. Abrogated ROS production through NAPDH inhibition reduced infiltration of peripheral immune cells and ameliorated EAE[177,178]. In contrast, intracellular ROS production from CNS macrophages is induced by phagocytosis, and phagocytic clearance of myelin is suggested to demand ROS[179,180]. The effects of oxidative stress are, however, complex, and the MS drug dimethyl fumarate is shown to, besides its NRF2 activating abilities, also to increase peripheral oxidative stress in an immunomodulatory effect[181] .

Re-myelination is regulated by myelin debris-clearance but also by cytokines secreted by glial cells and immune cells. The differentiation of OPCs and thus re-myelination is inhibited by TNF, IL-17, and IFN-γ found in MS lesions and produced by microglia, although they are not considered the primary source[182-184]. Macrophage NLPR3 inflammasomes are found abundant in lesions of RRMS and primary progressive MS where IL-1β is a candidate biomarker for poor prognosis and possibly a treatment target[185]. In EAE, IL-1β has an ambiguous role, damaging through recruiting and polarizing T-helper cell phenotypes driving neuroinflammation. However, IL-1β also attracts and stimulates differentiation of OPCs and thus exhibits a positive impact on re-myelination[186-189]. This is partly mediated through the induction of microglial secretion of LIF and IGF-1 acting on OPCs[188,189]. However, this is a complex issue, and IL-1β is also shown to delay re-myelination in the Cuprizone de-myelination model[185].

Microglia are found early in MS lesions and participate in the recruitment and polarization of pathogenic peripheral immune cells by being a cytokine-source of, e.g., CXCL13 recruiting B-cells to the CNS, IL-1β and IL-23 polarizing pathogenic T-cells and allowing for monocytes to enter the CNS[17,23,24,186]. All with a crucial impact on EAE and correlating to MS disease[14,16,17,19,24,190]. The TAK1-NfKB axis regulates microglial cytokine expression that is a prerequisite for immune cell infiltration and de-myelination in developing EAE[104]. These events are at the crossroads of the “inside-out” vs. “outside-in” theories, where the latter represent the established idea that the event initiating a relapse is peripheral immune cells evading tolerance and entering the CNS[191]. The alternative “inside-out” theory claims that myelin damage is the initial event followed by microglial activation and recruitment of peripheral immune cells, accelerating the damage[192]. Support for this comes from animal studies with induced oligodendrocyte or myelin damage leading to inflammatory relapses and rare mutations in a myelin component gene associating with MS[192,193].

1.6 Ageing microglia

The Ageing of an individual is not a uniform process in all its organs and cells. Microglia are cells with low mitotic activity, which keep the telomeric length but, on the other hand, opt out on the possibility of a fresh start as the cells accumulate misfolded proteins and leaky mitochondria[194]. Age is associated with an altered immune system, dysfunctional myeloid cells, neurodegeneration, and for MS patients associates with a switch to progression[194-196]. The core homeostatic microglial phenotype of adult mice in healthy conditions is also well represented in aged mice and humans, and the transcriptomes overlap between the species[97,197,198]. However, in both mice and humans, advanced age is associated with alterations in microglial subpopulations where transcriptomes indicate reduced signaling of the TGF-β pathway and increased expression of genes involved in vesicle biogenesis and phagocytosis, including *APOE*[197,198]. Despite this, functional microglial phagocytosis of protein aggregates and myelin debris decline with age while the lysosomal burden increases indicating either a dysfunctional degradation or higher degradational demand[199-201]. The aged microglia accumulate leaky ROS-producing mitochondria and display NLRP3 inflammasome instability, both pathogenic in progressive MS and neurodegeration[185,202,203]. The age-associated changes in lysosomal degradation and ROS production associate with a so-called Lipofuscin accumulation of highly oxidized protein aggregates that in microglia cause further defects in metabolism, inflammatory polarization, altered morphology, and reduced survival[77,200,204,205]. Of note, these phenotypical changes and the microglial senescence could be derived from an age-associated autophagy impairment, which is discussed in depth later.

As a cure for an age-associated decline in microglial function, an aged microglia replacement is proposed. In experimental models, the repopulating microglia had reduced cellular and tissue ageing features, including a lowered load of lipids and lysosomes and reduced *Apoe* expression[77,206-208]. The re-established microglia is further shown to differentiate accordingly to CNS-region defined cues[208,209]. The clinical impact from a true microglia replenishment of the myeloid CNS niche in EAE and MS remains to be evaluated.

1.7 Infiltrating and border-associated-myeloid cells

Microglia are the only CNS parenchymal myeloid cells in homeostatic conditions, but macrophages of other origins populate the CNS interfaces[106]. These populations, collectively referred to as BAMs or CAMs (Border or CNS Associated Macrophages, respectively; hereafter referred to as BAMs), reside in the perivascular space, the choroid plexus, and in the meninges. By fate-mapping, these cells are suggested to derive from an embryonic CD206+ population giving rise to BAM, while the CD206- is the origin of the TGF-β dependent microglia population[210]. Similar to microglia, BAMs rely on the transcription factors PU.1 and IRF8. These cells are self-renewing in homeostatic conditions, while depletion through CSF1R-blocking resulted in persistent contamination with bone marrow-derived myeloid cells[210,211].

The BAM populations all have transcriptomes disparate from microglia except a small subset of macrophages residing in the choroid plexus[210,211]. This population shares the transcriptional signature with TREM2 derived DAM, suggesting the existence of a non-parenchymal microglia-like cell, possibly the cell known as “Kolmer’s epiplexus cell”

described first in 1921[211,212]. As with other DAM´s, these cells have enriched expression of genes involved in phagocytosis and lipid metabolism, including *Clec7a* and *Apoe,* although in homeostatic conditions[211]. The BAMs are all targeted in the CX3CR1 inducible CRE model, but compared to microglia, they constitute tiny populations with no solid evidence of impact on MS and EAE, although they respond to experimental neuroinflammation by altered expression patterns[106,213]. However, the meninges are considered a niche for lymphoid cells in progressive MS, and the choroid plexus is suggested to be a route of entry of T-cells in MS, implying these populations to influence a disease pathology not fully covered by animal models[214,215].

The inflamed CNS tissue in MS and EAE is infiltrated by classical and nonclassical monocytes differentiating into monocyte-derived dendritic cells and macrophages. Monocyte-derived DCs have an EAE-specific signature with, e.g., elevated expression of *Clec9a* coding a receptor binding necrotic cells for processing and cross-presentation[106,216]. The monocyte-derived macrophages are distinguished from microglia by, e.g., surface marker CD49d or high expression of CD44, CD45, or F4/80 and are functionally less health-promoting phagocytes during EAE[18,78,106,110,114,217]. Although microglia have pathogenic potential, executive functions in T-cell activation, de-myelination, and axonal damage largely depend on CCR2+ and/or Ly6C+ infiltrating bone marrow-derived myeloid cells[18,114,186,218-220]. In EAE, these cells egress from bone-marrow and enter the CNS in a CCL-2 and GM-CSF dependent manner[18,21,186,219-222]. The pluripotent cytokine GM-CSF is found abundantly in MS lesions and demanded in EAE induction, and overexpression causes spontaneous neuroinflammation with infiltration of inflammatory monocytes[20,21,186,221,222]. Of note, these infiltrating myeloid cells do not seem to contribute to the CNS parenchymal myeloid population once the autoinflammatory EAE relapse is ceased[220]. The “M1” and “M2” macrophage nomenclature has not survived the complexity added by methodological advances but assigned “M2” bone marrow-derived macrophages such as CD206+ ARG1+ IGF1+ cells are associated to re-myelination, and transfer of TGF-β stimulated macrophages ameliorates EAE[119,223].

Genes involved in antigen presentation, including the pivotal risk allele HLA DRB1*1501, are the strongest risk factors for MS where T-cells activated by APCs are pathognomonic[3]. Microglia are known to be weak in APC functions in stimulating and polarizing T-cells, a function rather executed in the CNS in MS and EAE by monocyte-derived DCs and macrophages[106,224-227]. Before entering the CNS, T-cells are activated in lymphoid tissues and/or possibly by the BAMs or DCs in the choroid plexus[214,226,228].

We can conclude that the myeloid CNS landscape can be far more complex and adaptive than previous notions, with functions defining subpopulations and associations to pathology to be discovered.

1.8 AUTOPHAGY

1.8.1 Canonical autophagy

Canonical (macro)-autophagy is by definition a katabolic process degrading errant or superfluous intracellular proteins or organelles in an endosomal-lysosomal route. This process uses a set of proteins, commonly prefixed ATG, with defined functions at the different autophagosome formation steps. While the energy state of a cell and its surrounding tissue is the foremost regulator of autophagy, many other intra- and extra-

cellular stimuli, including CSF1R, TREM2, and C-type lectin receptor engagement, also regulate myeloid cell autophagy[75,229-231]. The launching step of autophagosome formation is the pre-initiation complex assembly, a process induced by AMPK kinase activity and regulated negatively by Akt by activating the mTOR-complex. These pathways are regulated downstream the CSF1R and TREM2 and induce autophagosome maturation in myeloid cells by activating ERK1/2[66,128,130,232,233]. TREM2 is also shown to inhibit aberrant mTOR-mediated autophagy in a model addressing this receptor as a risk factor for Alzheimer´s disease[229]. The aged microglia is shown to depend on alterations in mTOR-signaling in acquiring the age-associated phenotype both in human and animal models[234].

In canonical autophagy, the ULK1/2 acts as a regulated and regulating node acting downstream by phosphorylating core-autophagy proteins, including activation of the BECLIN1/VPS34 nucleation complex leading to formation the phagophore membrane[235]. With the vesicle membrane in place, the cargo for degradation is loaded in either a selective or non-selective process. The phagophore maturation to a double-layer autophagosome requires ATG7 activation of the ATG5-ATG12 complex and a cysteine residue's lipidation on MAP1LC3 (commonly referred to as LC3-II when lipidated) exposed by the ATG4 kinase. LC3 is moreover suggested to have an additional role in the selection of cargo[236]. The LC3-II coat the autophagosome and is used as the standard defining marker of autophagy. A RAB GTPase and SNARE protein mediate the fusion with a lysosome that finalizes the degradation of the autophagosome's and its cargo[237]. By being the main route of degrading various cellular components, autophagy is instrumental in cellular plasticity and survival, emphasized by the findings that mice carrying *Ulk1/2, Atg5,* or *Atg7* constitutive gene deletions are neonatally lethal[238].

Autophagy is generally considered to promote survival and differentiation in immune cells, while inhibition is associated with proliferation, including malignant myeloproliferation[239,240]. As many of the immune system mediators regulate autophagy, autophagy also controls immune responses by regulating the density of immune receptors and providing peptides for antigen-presentation exemplified by a model where *Atg5* deficiency specifically in dendritic cells ameliorates EAE[228,241,242]. Autophagy directly regulates inflammasome stability and secretion of IL-1 superfamily cytokines, where IL-1β is well known in MS and EAE. Further, impaired canonical autophagy is entailed to harmful inflammatory processes, including ER stress and defective clearance of mitochondria causing dysregulated ROS production and increased Lipofuscin load[204,243-247]. Lipofuscin associates with cellular senescence which impairs function and reduces microglial density in chronic MS lesions[13,248].

1.8.2 Non-canonical autophagy and phagocytosis

Non-canonical autophagy is a loose definition of cellular vesicular formations using parts of the autophagy protein machinery. This nomenclature includes activities not traditionally addressed as autophagy, such as microbe degradation in “Xenophagy” and ATG dependent secretion of cytokines. In a process referred to as autophagy- or LC3-associated phagocytosis, myeloid cells load dying cells, debris, or microbes into phagosomes labeled by LC3-II for degradation through the autophagosomal-lysosomal path[249]. This process is induced by binding to a selection of myeloid surface receptors, including CD11b, TLR4, and phosphatidylserine receptors, including Tim 3/4 and Fc gamma receptor - all myeloid

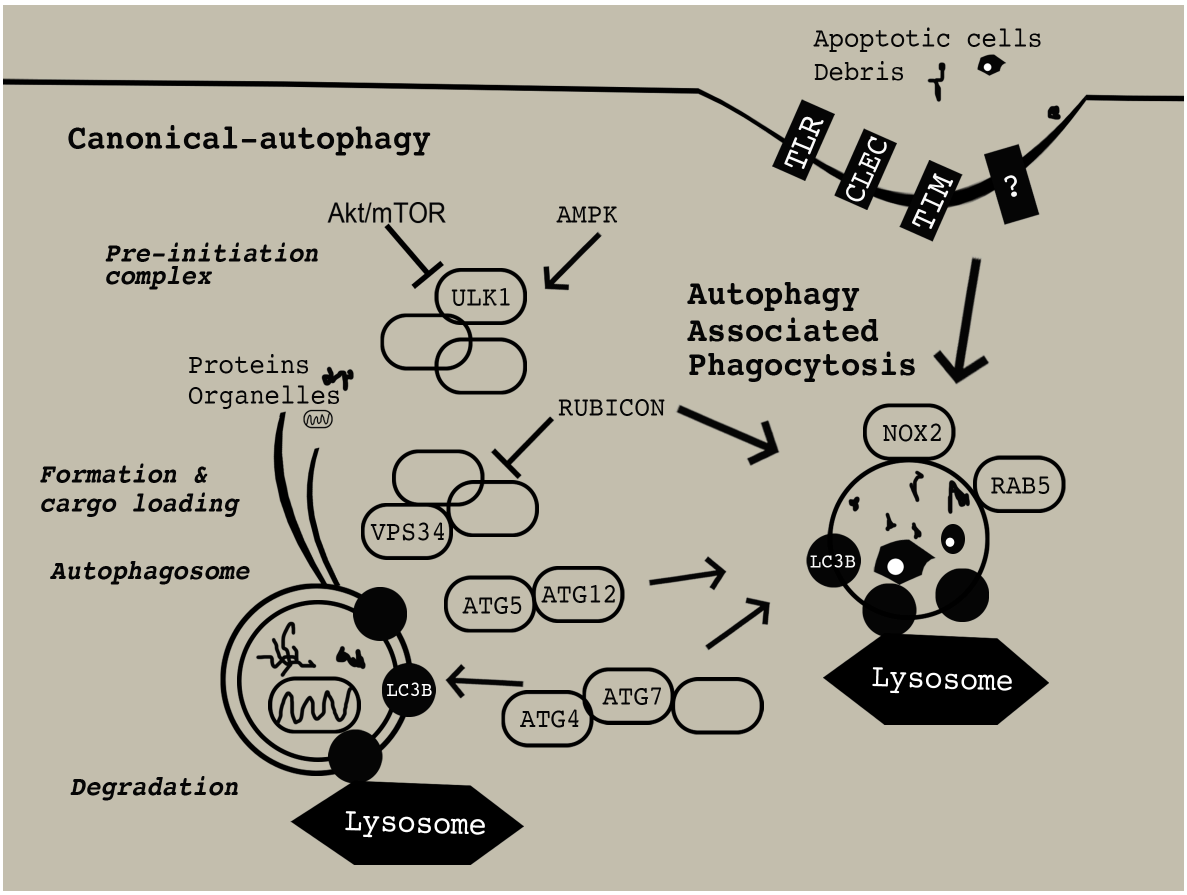
receptors implicated in neuroinflammation[250]. Further, the engagement of CLEC7A is known to induce LC3-associated phagocytosis during fungal infections[251,252]. Autophagy-associated phagocytosis is orchestrated by the protein RUBICON that simultaneously inhibits canonical autophagy by restraining VPS34[253]. This process is not regulated by mTOR and independent of the pre-initiation complex containing ULK1/2 but relies on the enzymatic activity of ATG7 in lipidation of LC3[249,253,254]. With the autophagy-defining lipoprotein embedded in the phagosome membrane, the lysosome fusion is facilitated, and phagocytosed content degraded[249,253,254]. In addition to LC3-II, degradation of this single-layer auto-phagosome require ROS production from the NOX2 complex[228,253]. Moreover, halted degradation of these vesicles also influences clearance capacity by impeding retromer trafficking of scavenger and regulatory receptors, e.g., CD36 and TREM2[134,255,256].

Impairment of the macrophage autophagy-associated phagocytosis is shown to aggravate inflammation and autoimmunity in an antibody mediated lupus-like disease and in models of autism, Alzheimer´s, and Parkinson´s disease with phenotypes derived from microglia deficiency in *Atg5* or *Atg7*[255,257-259]. In these models, pathology associates both with errant tissue clearance of proteins and debris and with other functional alterations of the targeted microglia and macrophages, e.g. cytokine secretion[253,255,257,260].

1.8.3 Autophagy in the pathology MS and other human diseases

Genetic alterations and mutations causing dysregulated or functional impairment of autophagy are found in several human pathologies ranging from asthma to malignancies. Among these diseases, we find several neurodegenerative conditions such as ALS, Parkinson´s, and Alzheimer´s disease, where many risk genes associated with impaired degradation of mitochondria and errant ROS production[261]. The accumulation of neuronal protein aggregates in neurodegenerative diseases such as Alzheimer´s, ALS, and Parkinson´s is suggested to be a result of dysfunctional autophagy-associated clearance[262-264]. In Crohn´s inflammatory bowel disease, an *ATG16L1* risk allele associates with increased mucosal inflammation[261,265]. This autoimmune disease entails an increased risk for developing MS and shows overlapping pathology to the one seen in the de-myelinated CNS[255,257]. Further, an impairment specifically in autophagy-associated phagocytosis is suggested in SLE pathology where an *ATG5* allele increases risk supposedly through errant clearance and processing in phagocytosis of dying cells[257,266].

In MS *ATG4D*, involved in LC3 labeling of autophagosomes in all forms of mammal autophagy, is detected as a risk gene for susceptibility[7]. Also, phagosome formation and maturation are detected as regulated pathways and *RAB5*, a GTPase important in phagosome maturation in, e.g., LC3 associated phagocytosis, have a highly significant risk-allele for MS incidence[7,253,255]. Autophagy regulatory mTOR, AMPK, Akt, and ERK pathways are also detected in pathway analysis of MS risk-genes[7]. Moreover, *CLEC16A* risk alleles in MS susceptibility associate mechanistically to MHC class II vesicular compartment maturation in a process suggested being autophagy dependent[261,267-270]. Finally, histopathological examination of microglia in MS lesions shows the increased density of autophagosomes and NOX2 activity, possibly linked to autophagy-associated phagocytosis[109,127].



Autophagy

1.8.4 Autophagy as a pharmaceutical target

Autophagy is a major house-keeping pathway in most mammal cells, and pharmaceutical regulation is applicable both in, e.g., stimulated degradation of hazardous proteins, inhibiting tumor cell survival processes, and modulating the immune response, including IL-1β and ROS secretion. However, targeting of an essential function such as autophagy could potentially increase the incidence of adverse effects[271]. The well-known autophagy-inducer rapamycin reverses the mTOR suppression on autophagy and acts as an immune suppressant preventing allograft rejection. Rapamycin also ameliorates EAE partly through ERK1/2 activation and is recently shown to alter the age-associated microglial phenotype[272,273]. Spermidine, found in, e.g., cheese, grains, and peppers, induces autophagy through downstream deacetylation of several ATGs and has been shown to increase lifespan in flies, worms, mice, and possibly humans mimicking benefits of calorie restriction[274,275]. In the MS-model EAE, spermidine alleviates clinical symptoms and pathology by polarizing monocytes to ARG1+ macrophages with reduced IL-1β secretion[276,277].

Another autophagy regulation goes through the activation of TFEB, a transcription factor stimulating autophagosome and lysosome component formation providing substrates for both canonical and non-canonical pathways shown protective in neurodegeneration and atherosclerosis through microglia and macrophage functions[278-281]. In these studies, TFEB activation is obtained upon treatment with trehalose, a sugar-molecules naturally occurring in, e.g., shellfish and mushrooms[279,282]. Trehalose is shown to induce autophagy through blocking surface glucose transport receptors on hepatocytes but how this associate with

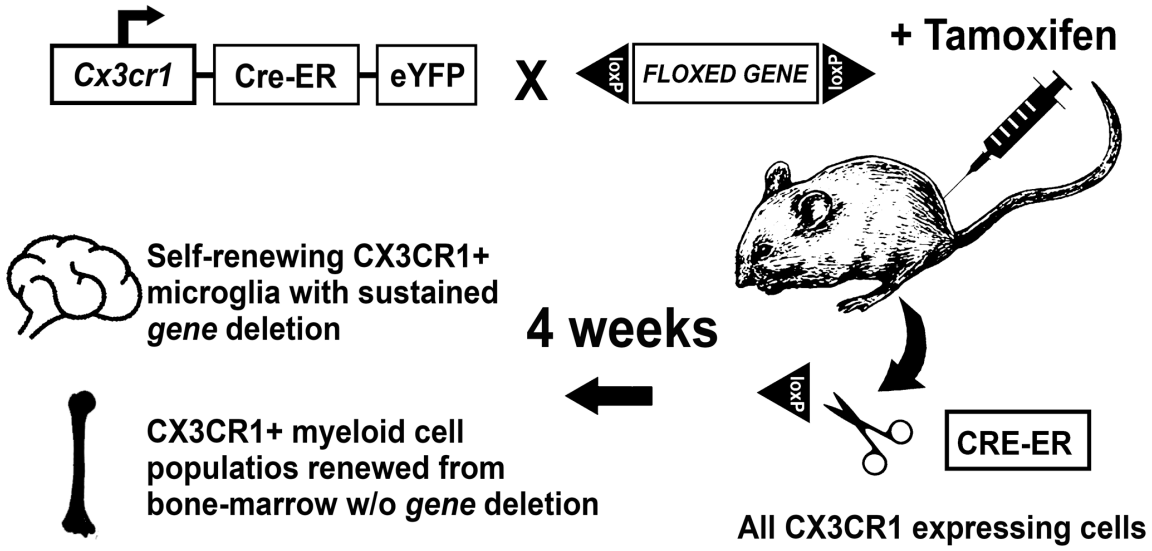
TFEB and its relevance in myeloid cells remains to be explored[283]. Like spermidine, trehalose is not only suggested to have therapeutic potential for specific pathologies but also to act in general for CNS health and long life[274,282,284]. Importantly, trehalose metabolism is found associated with MS through a risk allele of the gene coding for the trehalose degrading enzyme trehalase[7,285].

The genetic MS-associations indeed put the autophagy pathway and potential influence of environmental factors in the form of trehalose in an excellent position for further work, perhaps also therapeutically.

2 METHODOLOGICAL CONSIDERATIONS

2.1 Mouse Cre-Lox models

All experiments in **Paper I**, **II**, **III** were conducted using C57BL/6 mice. Gene ablation was accomplished using mice with cell-specific expression of the CRE-enzyme excising targeted genes flanked by lox(P) sites, referred to as Flox/Fl/fl. In homeostatic conditions, Cre expressed under the *Lyz2* promoter targets primarily bone marrow-derived myeloid cells, and surprisingly also neurons[286]. Microglial *Lyz2* expression and Cre-derived deletion are increased during inflammation[106]. Tamoxifen-induced *Cx3cr1*^{CreERT2} (hereafter referred to as *Cx3cr1*^{CreER}) model has a high recombination frequency in the microglial population but is only specific when bone marrow-derived populations are replaced from the periphery, which is not necessarily the case for BAMs[210,287,288]. Considering this and the possible impact on phenotype from Tamoxifen itself, experiments were conducted at least 4 weeks after the last Tamoxifen treatment. In this strain, CX3CR1^{CRE} is fused with a yellow fluorescent reporter (eYFP) reporter.



Microglial targeting in the Cx3cr1^{CreER} *model*

2.2 Diphtheria-toxin mediated depletion of microglia

Paper III is an application of thoroughly explored possibilities of depleting the microglial population[78]. The most successful depletion was accomplished by *Cx3cr1*^{CreER} mediated deletion of a stop cassette in Rosa26^{DTA} mice, leading to cell-specific diphtheria toxin

expression, which induces apoptosis and depletion of >99% of CNS myeloid cells. This could be maintained by Tamoxifen treatment-induced Cre expression. By transferring *Lyz2*^{Cre}*Tgfbr2*$^{fl/fl}$ *or Cx3cr1*^{CreER} *Tgfbr2*$^{fl/fl}$ to irradiated *Cx3cr1*^{CreER}*R26*^{DTA} recipient mice creating a chimera, a depletion was combined with a specific gene deletion in the monocyte and BMDM populations.

2.3 The EAE model

The EAE model employed in these studies was induced by active immunization with mouse recombinant MOG 1-125aa protein. The de-myelination occurs primarily in the spinal cord upon infiltration of peripheral immune cells and activation of microglia. This pathology includes inflammation, de-myelination and axonal damage/neuronal death. The induction of active EAE in C57BL/6 requires Freund´s complete adjuvant containing *Mycobacterium tuberculosis* which leads to pattern recognition receptor activation and amplification of the immune response. Immunization is accompanied by intraperitoneal injections of *Bordetella pertussis* toxin, which induces myeloid cell IL-1β secretion and possibly regulate BBB permeability[19,289-291]. In **Paper I** and **III**, mice of advanced age were used. Aged mice are known to have heterogeneous phenotypes and develop aggravated EAE upon MOG immunization.

2.4 Microglial phenotyping in vivo and in vitro

In our studies, dynamics of microglial phenotypes were evaluated by transcriptional analysis, ELISA cytokine quantification, flow cytometry, functional assays, and immunocytochemistry. Disease processes were examined and visualized by immunohistochemistry of CNS with microglia and macrophages at the site of pathology. Tools for studying specifically microglia and exclude bone marrow-derived macrophages are still developing. While microglia in flow cytometry can be defined as CD45^{Intermediate} CD11b+ Ly6G- under homeostasis, in the inflamed CNS no definite microglial marker for immunohistochemistry analysis is available. Healthy conditions offer, however, several microglia-specific markers such as TMEM119, SALL1 and P2RY12. Of note, the microglia signature is rapidly lost when cells are extracted from the CNS and cultured in vitro as they acquire an activated age-associated phenotype[292]. Time in culture and in vitro experiments were due to this kept to a minimum.

2.5 Autophagy monitoring and phagocytosis assays

In **Paper I** and **II,** autophagy was targeted by deletion of core autophagy genes *Ulk1* and *Atg7. Ulk1* has high homology with *Ulk2* also expressed in microglia, but the up-, and-down-stream regulation differs between the paralogs[60,61,293]. The consequence on autophagocytosis from these deletions was assessed as, e.g., IL-1β secretion, mitochondrial load, and LC3-II+ autophagosome densities after starvation phagocytic intake. Myelin-containing phagosomes targeted for degradation through the autophagy-lysosomal pathway were detected by membrane-bound LC3-II in an analysis where a mild permeabilization protocol allowed for unbound LC3-I to diffuse from the cytosol. In **Paper I**, we designed an experiment for flow cytometry to analyze the intracellular localization of phagocytosed content. Myelin or apoptotic cells were conjugated to one dye fluorescent in low pH

(lysosomes) and one emitting in a pH-independent manner (vesicles or cytosol). These experiments and most phagocytosis quantification detect uptake as intracellular content such as myelin or bacterial compounds, which is misleading regarding phagocytic clearance if the degradation of phagosomes is errant. To address this, we measured the capacity to clear myelin debris by exposing microglia and bone marrow-derived macrophages to fluorescent myelin, followed by quantification of the remaining myelin in the culture medium.

2.6 Analysis of RNA sequencing data

In **Paper I** and **III**, comprehensive expression analyses of RNA sequencing data were used to phenotype microglia. Differentially expressed genes (DEGs) were analyzed and visualized by detection of regulated pathways and pathologies in Ingenuity pathway (IPA) analysis, REViGO, and Over-representation analysis (ORA). We further employed a Gene Set Enrichment Analysis (GSEA) to investigate differences in expression between strains regarding predefined expression signatures. This was employed to compare our sequencing data to DAM-subpopulation signatures defined by single-cell RNA sequencing of microglia in, e.g., MS and Alzheimer's disease models. However, our experiments aimed not to identify new subpopulations that arise in the models deficient in targeted genes but rather to contextualize the general shifts in microglial phenotypes.

3. ETHICAL CONSIDERATIONS

For obvious reasons, CNS tissue from humans is not easy to access, and animal experiments have been instrumental in finding cues in treating and understanding neurological disease and treatment targets. Experimental animal research entails a responsibility to ensure quality and careful planning to reduce suffering. I have, through the Ph.D. education kept the use of animals to the minimum without hampering statistical significance. During the later parts of my education, I refined my technical skills and could use the same animal for several experimental analyses. To replace in vivo and ex vivo experiments was not possible due to the tissue-dependent nature of targeted microglial cells. All experiments have been conducted according to the ethical permits.

4. AIMS

Paper I

To characterize how impaired autophagy by gene ablation or age-associated decline influence cellular phenotype and clinical EAE. We further aimed to explore if this pathway could be targeted pharmacologically to ameliorate disease.

Paper II

To investigate if microglia depend on autophagy in maintaining a CNS protective population during ageing.

Paper III

To study if peripheral cells depend on TGF-β in the process of integrating to the microglial CNS niche and phenotype.

5. RESULTS

5.1. Paper I

Previous studies by our group associated reduced *Atg7* expression in immune cells to aggravated EAE in an unbiased approach in rat-strain crosses[294]. We induced *Atg7* deficiency in T-cells under the *Lck* promoter and myeloid cells under the *Lyz2* promoter in a mouse Cre-Lox system. The deletion in T-cells had minor effects on $CD8^+/CD4^+$ T-cell proportions in lymph nodes and did not affect clinical EAE. The *Lyz2*-CRE driven *Atg7* deletion was associated with a loss of recovery from EAE. Since *Lyz2* is expressed in many of the myeloid cells involved in EAE, we quantified *Atg7* expression in different subsets and found *Atg7* highly expressed and regulated in the CNS resident myeloid cell microglia. We also found deletion of floxed *Atg7* in microglia under the *Lyz2* promoter to be elevated during inflammation. To further explore this, we generated a model with microglia-specific deletion of floxed *Atg7* using the $Cx3cr1^{CreERT2/+}$ (hereafter referred to as $Atg7^{fl/fl}Cx3cr1^{Cre\mathrm{ER}}$), which have the benefit of an inducible deletion. This is important since autophagy has many key functions in the development, differentiation, and survival of the cell, tissue, and individual.

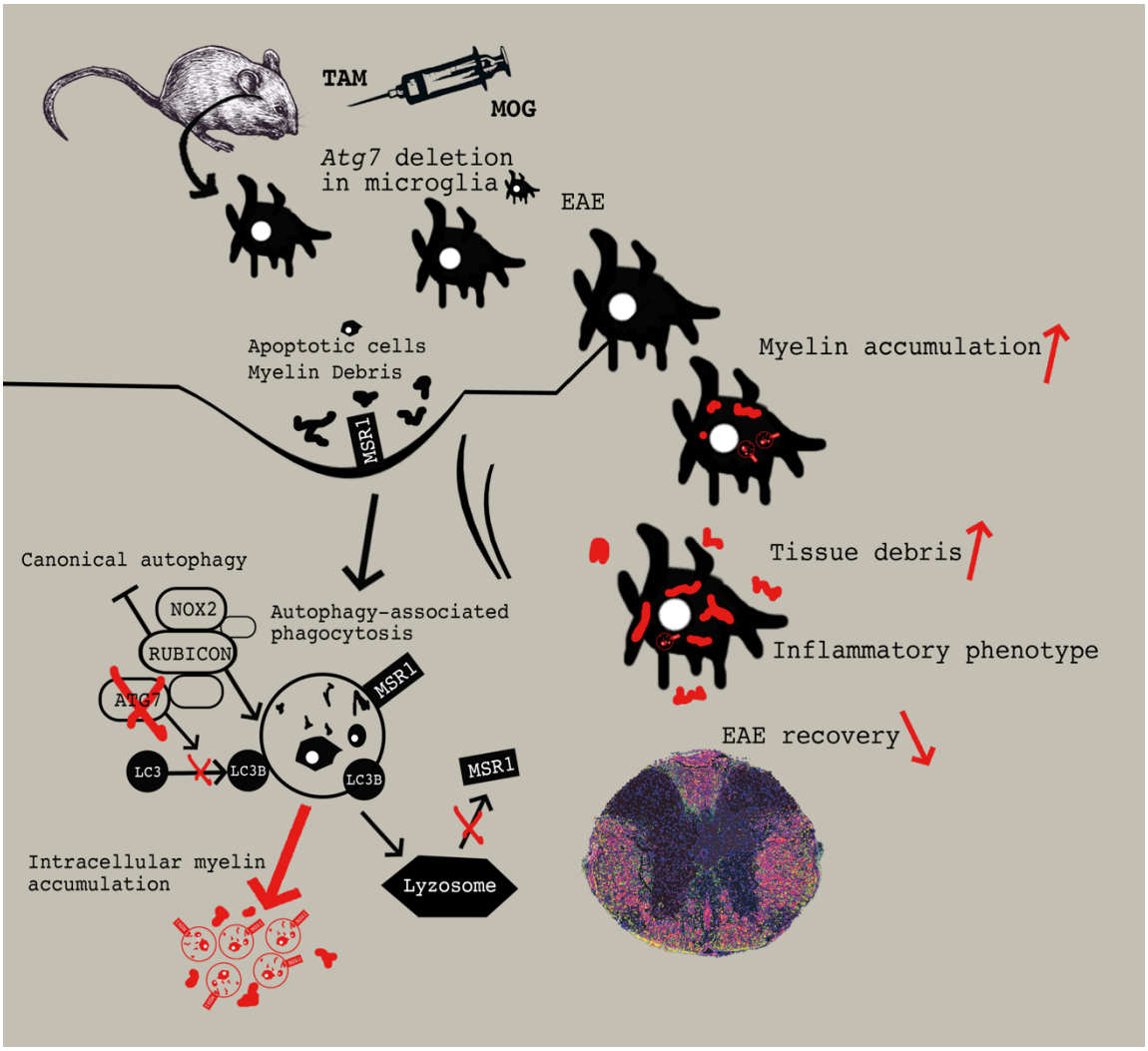
The clinical EAE in the $Atg7^{fl/fl}Cx3cr1^{Cre\mathrm{ER}}$ reproduced the chronic course seen in the $Lyz2^{\mathrm{CRE}}$ model, supporting a notion of a microglia-derived phenotype in both strains. In MS and EAE, phagocytic clearance of dead cells and myelin debris is suggested to be a microglial duty, and microglia lacking *Atg7* had accumulations of intracellular myelin. Compared to Wt, we found the myelin-containing phagosomes in autophagy-impaired microglia to have reduced LC3-II labeling and impaired loading into low-pH lysosomes. This concludes a dysfunctional phagocytic degradation through LC3-/autophagy-associated phagocytosis, a process previously known in macrophage context in models of SLE and Alzheimer´s disease [255,257]. This process was discriminated from canonical autophagy by employing deletion of *Ulk1*, an autophagy protein not demanded in autophagy-associated phagocytosis. Further, the expression of the master regulator of autophagy-associated phagocytosis *Rubicon* was found upregulated early during EAE, which supported the importance of this process. In a myelin clearance assay, we aimed to explore if the impaired degradation affected the uptake and hence clearance of tissue debris. The *Atg7*-deficient microglia had a rapidly exhausted capacity of myelin clearance, and after exposure to myelin debris in vivo and in vitro, they accumulate not only the content but also myelin scavenger receptors MSR1 (SCARA1, CD204, SR-A) and CD36. We further validated this path by detecting MSR1 blockade to inhibit myelin uptake and blocked lysosomal fusion to reduce receptor-recirculation.

Analyses of EAE CNS detected an increased intracellular myelin load in microglia lacking *Atg7* accompanied by an increased load of tissue myelin debris and consequential reduction in OPC differentiation to myelinating cells. During EAE, the $Atg7^{fl/fl}Cx3cr1^{Cre\mathrm{ER}}$ mice had increased immune cell infiltration to the CNS, including bone marrow-derived macrophages. However, these cells could not compensate for the reduced phagocytic capacity of microglia, supporting the idea of microglia as the primary myelin phagocyte during CNS inflammation. RNA sequencing of microglia sampled during EAE associated a DAM signature to the *Atg7* deficiency. Accordingly, we found upstream DAM regulators TREM2 and *Apoe* highly expressed in $Atg7^{fl/fl}Cx3cr1^{Cre\mathrm{ER}}$ microglia during EAE. We could also define and validate true subpopulations based on CLEC7A, one of the DAM signature

genes. The CLEC7A^{High} population was increased in counts in the CNS of *Atg7*$^{fl/fl}$*Cx3cr1*^{CreER} mice and possessed this model's highlighted impairments, including accumulated intracellular MSR1. Pathway analysis of microglia during EAE revealed activated inflammatory pathways detected as enriched IFNγ and GM-CSF signaling in *Atg7* deficient microglia at day 21 p.i. The later day 35 p.i. timepoint showed regulation of, e.g., metabolism and oxidative stress pathways associated with progressive MS[3,5,115].

Autophagy is a process known to decline with age, which also is a strong risk factor for progressive MS. As discussed above, several genes implicated in autophagy and phagocytosis have allele variants associating with MS risk[7]. By treating aged mice with trehalose, a sugar molecule found in, e.g., plants, fungi, and shellfish, we could reverse the aggravated EAE associated with age in a microglia-dependent fashion. Trehalose induced TFEB nuclear transfer and thus transcriptional activity, increasing the biogenesis of autophagosomes and lysosomes. Our experiments found a specific effect on autophagy-associated phagocytosis as the effect was not abrogated by ULK1 deficiency.

This study shows how microglia regulates EAE recovery through phagocytosis of myelin and associated phenotypic alterations. We also showed how age-associated impairments in this process could be reversed by a dietary sugar compound potentially affecting a pathway untargeted by available medications and possibly of certain importance in age-associated progressive MS.



Graphical abstract for Paper I

5.2 Paper II

As age entails altered canonical-autophagy and microglial phenotypes, we sought to examine this in the context of CSF1R signaling and neuroinflammation[4,234,295,296]. In the aged CNS (>20 months), we found microglia to have a reduced proliferation tied to an increased cell survival indicating a slower turnover compared to adults (<5 months). While CSF1R, the main receptor for microglial survival and proliferation, had reduced surface density on aged microglia, the corresponding ligands CSF-1 and IL-34 had elevated expression in the aged CNS. By analyzing known phosphorylation-sites downstream of CSF1R engagement, we found aged microglia to have increased activating phosphorylation of ERK1/2, AMPK, and a reduced activating Akt phosphorylation. Consequently, aged microglia were significantly more sensitive to ERK1/2 inhibition. AMPK induces canonical-autophagy through the mTOR and pre-initiation autophagosome complex, including ULK1 activation, an effect inhibited by activated Akt. ULK1 activation is known to activate ERK1/2 and to be important in cellular survival[243,297]. Knowing this, we generated mice with a specific microglial deletion of floxed *Ulk1* upon Tamoxifen induced CRE-expression under the CX3CR1 promoter (hereafter referred to as $Ulk1^{fl/fl}Cx3cr1^{CreER}$). In adult mice, the microglia population kinetics was not affected upon this deletion, but after about 2 years, the population was significantly reduced by about 40%. Importantly, this reduction was not compensated by infiltration of other macrophages.

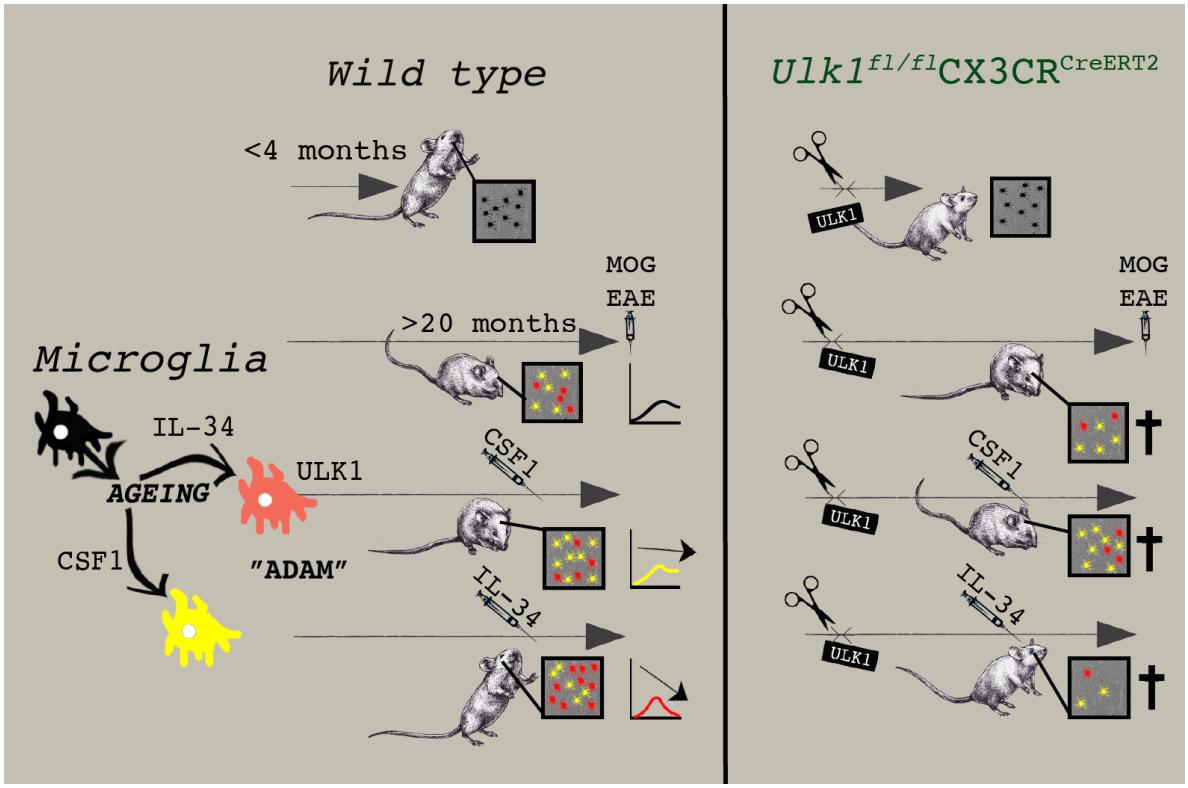
We traced the diminished microglia specifically to a loss of the highly activated ERK1/2 population and that ULK1 deficient microglia suffered from increased ER-stress associated with apoptosis. The loss was most prominent in the cerebral cortex. This chain of autophagy impairment and apoptosis has previously been described in other cells[243]. ERK1/2 is suggested to be an upstream regulator of DAM microglia characterized by a reduced CSF1R, increased CSF-1, and further by subpopulation markers such as the MHC class II, TREM2 and CD11C (ITGAX) [272,298]. In the autophagy-dependent microglia population with highly activated ERK1/2, these markers were found at a higher density, supporting an activated DAM subpopulation attribute.

In order to expand this Autophagy Dependent Age-acquired Microglia, referred to as "ADAM", we treated aged Wt and $Ulk1^{fl/fl}Cx3cr1^{CreER}$ aged mice with intracisternal CSF-1 and IL-34 injections. CSF-1 treatment evoked in both strains a large increase of the microglia less autophagy-dependent and reduced activation of ERK1/2. Conversely, the IL-34 treatment caused a substantial expansion of the ADAM population. This IL-34 induced expansion was specifically canceled in the *Ulk1* deficient mice revealing an aged microglial IL-34/CSF1R/ULK1 axis. Notably, we did not see a significant infiltration of peripheral immune cells in any strain or treatment condition.

The absent ADAM and the reduced microglia population without peripheral infiltration revealed a unique opportunity to study the influence of microglia on disease pathology in the MS model EAE, which is known to aggravate with age. We found the EAE in aged mice deficient in ADAM to associate with high mortality not rescued by IL-34 or CSF-1 treatment even though the latter has similar total microglial counts as aged Wt mice. By quantifying neurons and glial cells by number and apoptosis markers, we found the loss of the ADAM population to associate with a significant reduction of OPC, OLs, and neurons as well as microglia themselves during EAE. Upon IL-34 induced expansion of the ADAM in aged Wt mice, we detected a significant reduction in clinical scores and

increased survival of microglia and CNS cells during EAE. We could also reproduce a neuroprotective effect from IL-34 treated microglia in vitro and detected several neurotrophic factors increased in expression in microglia upon this specific stimulation.

This study discovered and characterized a CNS protective microglial population acquired with age defined by a demand for autophagy protein ULK1 and activated ERK1/2. The absence of this population is associated with aggravated disease phenotypes regardless of total microglial density. Importantly, this population expanded specifically upon IL-34 treatment, a finding to consider when targeting the CSF1R in modeling and treatment of microglia-associated pathologies.



Graphical abstract for Paper II

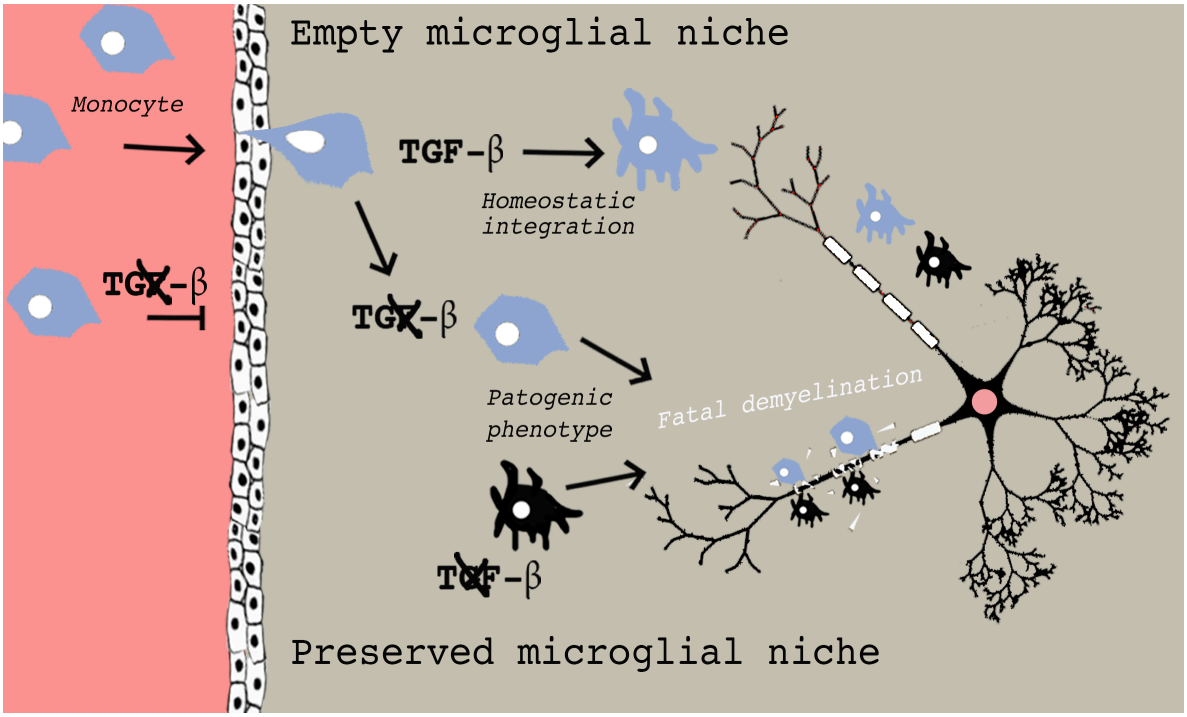
5.3 Paper III

TGF-β is known to be crucial in establishing a mature microglial homeostatic phenotype[98,99]. The microglial niche in the CNS parenchyma is, under inflammatory challenges and depletion, populated partly by infiltrating monocytes differentiating into bone marrow-derived macrophages. Our group's previous findings show how depletion is followed by competitive repopulation by microglial cells and monocytes and how these cells adopt shared phenotypes shaped by the tissue surrounding and an intrinsic signature defined by origin[78]. We found the deletion of the TGF-β receptor in *Lyz2* expressing monocytes to associate to reduced integration of bone marrow-derived cells into the CNS parenchyma after microglial depletion. Thus indicating a demand for this cytokine signaling in the migration process or survival and expansion in the target tissue.

In order to silence TGF-β signaling in the repopulated CNS myeloid cells, we utilized the finding of an upregulated *Cx3Cr1* expression in the infiltrating bone-marrow derived cells. This allowed us to employ the inducible deletion of floxed $Tgfbr^{fl/fl}$ upon Tamoxifen induced $Cx3cr1^{CreER}$ post infiltration to the CNS of bone-marrow-chimeric mice. The lack

of TGFBR2 in this population altered the transcriptome with upregulation of genes associated with antigen-presentation, inflammation and phagocytosis. About 12 days after gene deletion, the mice developed a progressive motor impairment and died around 10-25 days later. Immunohistochemistry revealed de-myelination and axonal dysfunction in association to myelin-laden giant macrophages in the dorsal spinal cord, causing fore- and hind-limb palsy and late-stage disease also affecting the brain. Without depletion, TGBFR2 deletion specifically in microglia caused similar pathology and symptoms but with later disease-onset compared with the monocyte repopulation model.

In conclusion, we show how bone marrow cells demanded TGF-β signaling to establish a CNS parenchymal myeloid cell and how this population, once settled, requires TGF-β to inhibit a pathogenic macrophage process. This is in line with the need for TGF-β for microglia during developmental establishment and maintenance of a homeostatic phenotype and could be employed in microglial/macrophage replacement therapy and to polarize these populations.



Graphical abstract Paper III

6. DISCUSSION AND FUTURE PERSPECTIVES

This thesis supports the notion of microglia as guardians of the CNS and how malfunctions of these cells cause pathology due to lack of health-promoting capacities. The microglial cell has been defined by localization and morphology for a century, but we still struggle to determine unique microglial signatures and features to distinguish them from bone marrow-derived macrophages. This and the obstacles CNS offer regarding tissue sampling in vivo are factors delaying the understanding of microglia in disease and a health-promoting context.

Microglia-specific gene targeting is still a struggle

The *Cx3Cr1*^{CreER} model targeting microglia is valuable but not completely decisive in its specificity and is likely to be contaminated by BAM. As an alternative, a *Hexb* promotor has been presented as microglia specific and stable in homeostatic and inflammatory conditions[299]. Another suggestion is to combine a myeloid promoter with a CNS specific, e.g., *Cx3Cr1* and *Sall1* with split Cre-fragments[287]. However, we should acknowledge the pitfalls of all models in excluding microglial subpopulations and/or including other immune- or CNS-cells.

Microglial depletion and replacement

Microglial replacement therapies upon, e.g., CSF1R inhibition are proposed to ameliorate degenerative and inflammatory neuropathology. Due to the accumulation of leaky mitochondria, impaired autophagy, accumulated mutations, and lipid inclusions, replenished microglia could substantially impact age-associated CNS disease. Our group's previous findings and the results presented in **Paper III** show that bone marrow-derived cells can contribute to a re-established myeloid CNS population following depletion and how TGF-β signaling is demanded and beneficial in maintaining a health-promoting cell in this microglia niche. This can be employed in a desirable treatment regime in polarizing a population that, once established, does not need further pharmaceutical support. **Paper III** further emphasizes previous findings that tell us that microglial origin matters and possibly that replacement therapies should actively inhibit colonization by myeloid cells of bone marrow origin. Given the troubles of depletion models regarding lack of specificity and undesired inflammation caused by cell death and/or toxins, our strategies of specific targeting of repopulating cells presented in **Paper II** and **III** is valuable for future work. In addition to the discovery of myeloid-derived pathogenicity shackled by TGF-β.

Up to date, there is no evidence of bone marrow-derived myeloid contribution to the CNS parenchymal myeloid cell population during homeostasis or even after the acute inflammatory challenge. Yet, we need to study this in a broader context investigating influence from ageing, chronic inflammation, and cytostatic or immune-modulatory treatments since a possible contribution of a bone marrow-derived cell likely affect pathology.

Disease-associated microglia – Loss of a health-promoting phenotype or intrinsic pathogenicity?

Microglia described as “activated”, “amoeboid,” and recently “DAM” have been addressed as pathogenic based on its context of inflammatory and neurodegenerative disease, and

largely by parameters adopted from phenotyping of bone marrow-derived myeloid cells, e.g., molecules associated to antigen-presentation. A likely more relevant idea is now emerging that activated microglia found in the inflammatory CNS lesions have designated functions in restoring a healthy CNS, where phagocytosis is a prominent feature shown in **Paper I** and by others[4,101,106,134,155,300]. This implies the defects in myelin processing and scavenger receptor recirculation associated with ATG7 deficiency shown in **Paper I** to be assigned a dysfunctional DAM rather than this population to have an intrinsic pathogenic phenotype. It is also possible that the impairment of the key phagocytic function causes an accumulation of this microglial subpopulation, defined by, e.g., CLEC7A density, on the cost of other subpopulations with features important in the recovery from inflammation. However, the DAM phenotypes are, for the most part, defined by single-cell RNA sequencing analyses and still need to be validated as spatially or functionally distinct populations or as transient responsive differentiation stages.

Autophagy-associated phagocytosis in MS and EAE

The list of suggested diseases with myeloid cell autophagy-associated phagocytic dysfunction is growing, and this pathway cannot be viewed as something irregular. One question that remains unanswered is if this is only a degradation pathway, or if the cargo selection has specific regulatory functions, and if the cargo by vesicle specifics is destined towards certain functions, e.g., antigen presentation. Another issue is how activation of this RUBICON-directed pathway affects canonical-autophagy functions during long-term exposure to, e.g., myelin or apoptotic cells. The process of autophagy-associated phagocytosis is also largely understudied in human disease, and findings by us in **Paper I** and others need to be reproduced or supported by studies on the pathogenesis of the human disease. This is also called for by the autophagy-associated genes and pathways detected in MS susceptibility genetics.

CSF-1 vs. IL-34 promoted microglial subpopulations in the aged CNS

The aged CNS was further explored in **Paper II** where we found a microglial population with activated ERK1/2, a known upstream regulator or the DAM microglia phenotype, to be dependent on canonical autophagy. This population referred to as “ADAM” was found protective during EAE, and loss of this population in the aged mice deficient in microglial ULK1 associated with high mortality, immune cell infiltration, and CNS cell death. Recent publications have explored the regional developmental disparate effects of IL-34 and CSF-1. We could expand the ADAM population specifically upon IL-34 treatment, which significantly reduced the signs of neuroinflammation compared to the CSF-1 treatment, which selectively expanded the autophagy-independent population with low ERK1/2 activation. We should also highlight that even though CSF-1 is associated with DAM and act on other cells than microglia, this treatment did not aggravate EAE disease. The evidently increased density of activation markers in the IL-34 expanded ADAM population supports a microglial DAM-phenotype with protective capacity. However, besides the IL-34 induced expression of neurotrophic factors the executive functions derived specifically from the two CSF1R ligands and to which degree they are redundant remains to be shown.

In conclusion, **Paper II** highlights the demand for canonical autophagy to maintain a microglial population with unique neuroprotective features during EAE. We can also view this model as a partial microglial depletion model, where the depletion in comparison to

other models is less effective but silent and not compensated by bone marrow-derived myeloid cells. How this affects the ageing CNS could be explored further in homeostatic conditions or other disease models, including exploring the suggested IL-34 specific effects.

The findings in **Paper II** revealing the age-acquired microglial subpopulation with higher demand for canonical-autophagy, suggesting this pathway rather more activated than declined with age. This is somewhat supported by an altered microglial phenotype downstream mTOR signaling recently reported[234]. However, in **Paper I**, we find an increased expression of DAM-associated genes in microglia deficient in both canonical and non-canonical autophagy shared partly with aged microglia. This further supports that the autophagy-dependent phenotype or subpopulation is acquired during ageing. One could also speculate an increased canonical-autophagy to occupy the autophagy proteins in aged microglia and thereby reduce the capacity for non-canonical autophagy associated phagocytosis — a deficiency we targeted by trehalose treatment in **Paper I.**

Induced autophagy as a potential therapeutic target in neuroinflammation

Loss of EAE recovery in the C57BL/6 model should probably be addressed as a prolonged inflammatory relapse rather than a transition to the progressive phenotype seen in human MS. This does not exclude shared specific pathways such as microglial autophagy. It is also possible that the aggravated EAE associated with age suffers from the same pathology as the progressive human disease. In contrast to models of neurodegeneration, studies in EAE addressing age is underutilized and likely informative.

The phenotypes presented in **Paper I** and **II** add to an emerging concept of autophagy as regulatory and executive function in myeloid cells. While immune-modulatory treatments commonly dampen the immune surveillance at large, autophagy induction tentatively has beneficial effects in surveillance of tumor cells and microbes and simultaneously dampening an aberrant immune activation. In general, boosting an immune function is rare in comparison to restraining immunity as a treatment regime. If this is due to complications or is an underused path remains to be seen.

The findings of trehalose ameliorating age-associated neuroinflammation in **Paper I** are promising and novel in targeting autophagy-associated phagocytosis. This could potentially be implemented as a dietary adjustment to reduce the risk of developing a disease or halt disease progression. However, deliberate trehalose supplementation should also be considered an option.

In this thesis, I present findings and ideas of health-promoting pathways in cells of the CNS microglial niche showing cellular phenotypes and functional outcomes. Knowledge of these cells is rapidly expanding and holds answers and offers solutions redeeming neuroinflammation that has been blindsided by methodological challenges.

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I arrived in the **Neuroimmunology unit close** to a decade ago. It was autumn, and I was a bit confused. The welcome I got from the **"old rat-gang"** was very warm and inclusive. From that era, I would like to address a few (of many) inspiring friends:
Melanie – Thank you for your sunny smartness and a perfect introduction to scientific labor and for handing over the "autophagy project", which still is my *Sagrada familia*.
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Until then.

Peace /Rasmus

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